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The differential effects of cell wall-associated phenolics, cell walls, and cytosolic phenolics of host and non-host roots on the growth of two species of AM fungi

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SUMMARY

Experiments were conducted to test the hypothesis that cellular compounds, especially wall-associated compounds, released during emergence of secondary roots, stimulate the growth of arbuscular mycorrhizal (AM) fungi. Purified cell walls, crude cell-wall extracts, crude cytoplasmic extracts, and phenolic compounds previously identified as cell wall-associated, from Ri T-DNA-transformed roots of host (*Daucus carota* L.) and non-host (*Beta vulgaris* L.) were incorporated into growth medium and tested for their effects upon growth of the AM fungi *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann and Trappe and *Gigaspora margarita* Becker and Hall. Purified cell walls of both plants had little effect on *G. gigantea* but non-host cell walls inhibited the growth of *G. margarita*. Ferulic acid, a major constituent of non-host root, depressed the growth of both fungi. Nothing tested which was unique to the non-host root affected hyphal growth to the point that contact would be prohibited. Caffeic acid, found in *D. carota* cytoplasm, also depressed growth of both fungi. Para-hydroxybenzoic acid, a constituent of *D. carota* roots, stimulated growth of *G. margarita* hyphae, but did not affect hyphal growth of *G. gigantea*. Vanillic acid, unique to *D. carota* root cell-wall extracts, stimulated hyphal growth and branching of both fungi, and should increase the probability of contact between fungus and host root.

Key words: *Gigaspora margarita*, *Gigaspora gigantea*, hyphal growth, phenolics, mycorrhiza.

INTRODUCTION

One reason that arbuscular mycorrhizal (AM) fungi are believed to be obligate symbionts is they have not been cultured axenically. Most research directed toward culturing these fungi has been in the study of root exudates which stimulate the growth of germinated AM fungus spores (Elias & Safir, 1987; Bécard & Piché, 1989). The role of flavonoids in microbe–host interaction is well documented (Phillips, 1992) and recent research has shown a stimulation of germ-tube growth by specific flavonoids (Bécard, Douds & Pfeffer, 1992; Chabot *et al.* 1992; Bel Rhlid *et al.*, 1993; Poulin *et al.*, 1993). These compounds, however, have resulted only in increased germination, hyphal growth, or production of auxiliary cells, not continued growth and production of new spores, and it is likely that they are unnecessary for the establishment of the symbiosis (Bécard *et al.*, 1995).

Observation of the dual-culture system of Ri T-DNA-transformed roots of *Daucus carota* and *Gigaspora margarita* (Bécard & Piché, 1992) suggests that initial infection by the fungus is most likely at sites on the primary root where secondary roots have started to emerge. Therefore, cell-wall constituents, such as phenolics, released during secondary root growth through the cortex and epidermis may act as signals or stimulants of hyphal growth.

In the work reported here, root cell walls, phenolics associated with root cell walls, and cytoplasm of host (*D. carota*) and non-host (*Beta vulgaris*) were tested for their effects on germinated

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spores of the AM fungi *G. margarita* and *Gigaspora gigantea*.

MATERIALS AND METHODS

Spores

Azygospores of *Gigaspora margarita* Becker & Hall (DAOM 194757) and *Gigaspora gigantea* (Nicol. & Gerd.) Gerdemann & Trappe were produced in pot cultures with *Paspalum notatum* Flugge in a greenhouse. Spores were collected by wet-sieving and centrifuging in 40% (w/v) sucrose and were surface-sterilized (Bécard & Fortin, 1988). Spores were germinated for experiments by aseptic insertion into Petri dishes of M medium (Bécard & Fortin, 1988) followed by vertical incubation at 32 °C in 2% CO_2 for 2–4 d.

Experimental media

Ri T-DNA-transformed roots of carrot (*Daucus carota* L.) and sugar beet (*Beta vulgaris* L.) were grown in M medium (Bécard & Fortin, 1988), in 0·2% (w/v) gelling agent (Phytagel®, Sigma) for 14 d at 24 °C. Roots were recovered by solubilizing the media in 25 mM Tris buffer containing 10 mM EDTA at pH 7·5 (Nagahashi *et al.*, 1993) before treatment and analysis. Crude cell-wall and cytoplasmic extracts were prepared as previously described (Nagahashi, Abney & Doner, 1996). Root tissue of 2–6 g f. wt was ground in liquid nitrogen and treated in a Parr nitrogen bomb to release cellular contents (Nagahashi & Seibles, 1986). Cell-wall and cytoplasmic fractions were separated by filtration and centrifugation. Each was exposed to alkaline hydrolysis and extracted in alcohol at pH 1·5 to release phenolics (Nagahashi *et al.*, 1996). Extracts were then evaporated to dryness under a stream of nitrogen, dissolved in 1 ml of methanol, and filter-sterilized into 300 ml autoclaved M medium before pouring plates. Commercial preparations of the phenolic compounds, caffeic acid, ferulic acid, vanillic acid and *p*-hydroxybenzoic acid, which were identified in extracts (Nagahashi *et al.*, 1996), were dissolved in either ethanol or water, filter-sterilized and transferred aseptically into M medium at specific concentrations to determine their effects upon growth of AM fungus spores. Control plates of M medium were amended with filter-sterilized ethanol (final concentration 0·001–1·0% (v/v) for 0·1–10 mg l^{-1} phenolic acid treatments, respectively) to account for effects of the solvent upon hyphal growth. Purified cortical cell walls (Nagahashi, Abney & Uknalis, 1994) were washed with bacteriocides (streptomycin sulphate, 0·2 mg ml^{-1} and gentamicin sulphate, 0·1 mg ml^{-1} final concentrations), followed by chloramine T (20 mg ml^{-1} final concentration), absolute ethanol and sterilized deionized-distilled water. The sterilized suspension of cell walls was added directly to the autoclaved M medium, thoroughly mixed, and plates were poured from the homogeneous suspension.

A single germinated spore was aseptically transferred within a core of medium to a square Petri dish (9 cm) containing either cellular extracts, phenolics, purified cell walls, or control solvent and incubated vertically as above for up to 20 d. Replicates (n = 4–7) of each treatment were prepared. The surface pH of the media (as determine by a surface electrode) at the end of the growth periods ranged from 5·3 to 5·6. Each experiment was run 2–4 times. Results of representative experiments are reported.

Fungal growth measurements

Overall hyphal length was measured by using a 2 mm grid and counting hyphal intersections (Newman, 1966). The primary germ tube (the main hypha, exclusive of its branches) was measured directly. Since the primary germ tube of both species exhibited a strong negative geotropic response, plates were turned when necessary to direct it away from the edges of the Petri plate. The number of hyphal branches originating from the primary germ tube and the number of clusters of auxiliary cells were counted directly under the dissecting microscope (×20 final magnification).

RESULTS

Crude cell-wall extracts and cytosolic extracts

The crude cell-wall extract from carrot roots had no effect on the growth of *G. gigantea* except to inhibit auxiliary-cell production (Table 1). The cytosolic extract inhibited total hyphal growth by approx. 22% but did not inhibit growth of the primary germ tube. Crude extracts from sugar beet cell walls inhibited the total hyphal growth by 28% and significantly reduced the average number of branches off the primary germ tube and the number of auxiliary cells relative to that of the controls. Although the crude extracts from the sugar beet cytosol had a slight inhibitory effect on the primary germ tube, the major changes were reduction of the total hyphal length of the number of branches off the primary germ tube, and of the number of auxiliary cells.

Sugar beet cell-wall extracts stimulated hyphal growth and branching of *G. margarita* (Table 2). Sugar beet cytoplasmic extracts and carrot cell-wall extracts tended to depress growth. In addition, carrot cell-wall extracts caused the normal unidirectional growth of hyphae to degenerate to a wavy or curly growth pattern.

Table 1. *Effect of host (carrot) and non-host (sugar beet) root cell-wall extracts and cytoplasmic extracts upon growth from spores of* Gigaspora gigantea *after 15 d**

Treatment	Hyphal length (cm) Total	Hyphal length (cm) Germ tube	Branches off germ tube	Auxiliary cells
Control	54·4±4·3	14·8±0·3	8·0±1·3	8·0±1·0
Carrot				
Cell wall	53·8±10·4	13·4±0·5	7·0±1·2	3·5±1·6
Cytoplasm	41·9±4·6	13·6±0·2	5·2±1·2	3·3±0·5
Sugar beet				
Cell wall	38·9±6·1	13·7±0·6	4·3±1·1	3·0±1·8
Cytoplasm	39·6±3·8	12·8±0·4	5·6±1·0	2·6±0·5

* Means of 4–7±SEM.

Table 2. *Effect of host (carrot) and non-host (sugar beet) root cell-wall extracts and cytoplasmic extracts upon growth from spores of* Gigaspora margarita *after 20 d**

Treatment	Hyphal length (cm) Total	Hyphal length (cm) Germ tube	Branches off germ tube	Auxiliary cells
Control	11·9±1·1	3·8±0·4	9·0±0·7	0·0±0·0
Carrot				
Cell wall	6·7±1·4	1·6±0·1	6·0±1·3	0·0±0·0
Cytoplasm	9·2±1·8	3·3±0·4	11·0±2·3	1·0±0·4
Sugar beet				
Cell wall	14·1±0·9	4·9±0·4	14·8±1·4	2·3±0·6
Cytoplasm	7·9±1·2	2·7±0·3	3·7±0·3	1·8±0·9

* Means of 4–7±SEM.

Table 3. *Effect of purified host (carrot) and non-host (sugar beet) cell-wall fragments upon the growth of hyphae from spores of* Gigaspora margarita *after 5 d**

Treatment	Total hyphal length (cm)	Auxiliary cells	Branches from primary germ tube
Control	14·2±1·0	3·7±0·8	13·9±1·1
Carrot	14·3±1·2	3·9±0·2	15·6±0·9
Sugar beet	8·9±0·7	2·7±0·2	3·5±0·5

* Means of 7–9±SEM.

Table 4. *Effect of phenolic compounds (5 mg l^{-1}) upon hyphal growth from spores of* Gigaspora gigantea *after 16 d**

Compound	Growth parameter (% of control) Hyphal length (cm) Total	Hyphal length (cm) Germ tube	Auxiliary cells
p-hydroxybenzoic acid	101±1	111±5	88±28
Ferulic acid	91±5	77±4	89±14
Caffeic acid	30±5	59±7	0±0
Vanillic acid*	145±11	105±2	110±28

Table 5. *Effect of phenolic compounds upon hyphal growth from spores of* Gigaspora margarita *after 17 d**

		Growth parameter (% of control)		
		Hyphal length (cm)		
Compound	Conc. (ppm)	Total	Germ tube	Auxiliary cells
p-hydroxybenzoic acid	1	118 ± 12	119 ± 7	67 ± 11
	2·5	120 ± 16	117 ± 11	50 ± 12
	10	162 ± 10	139 ± 5	133 ± 19
Ferulic acid	1	102 ± 4	91 ± 10	90 ± 33
	2·5	105 ± 16	119 ± 14	100 ± 27
	10	75 ± 7	84 ± 8	40 ± 10
Caffeic acid	1	75 ± 8	81 ± 2	88 ± 24
	10	74 ± 4	111 ± 15	112 ± 31
Vanillic acid	0·1	98 ± 6	189 ± 14	112 ± 17
	1·0	97 ± 5	132 ± 23	78 ± 11
	10	89 ± 14	139 ± 20	115 ± 13

* Means of 4–7 ± SEM.

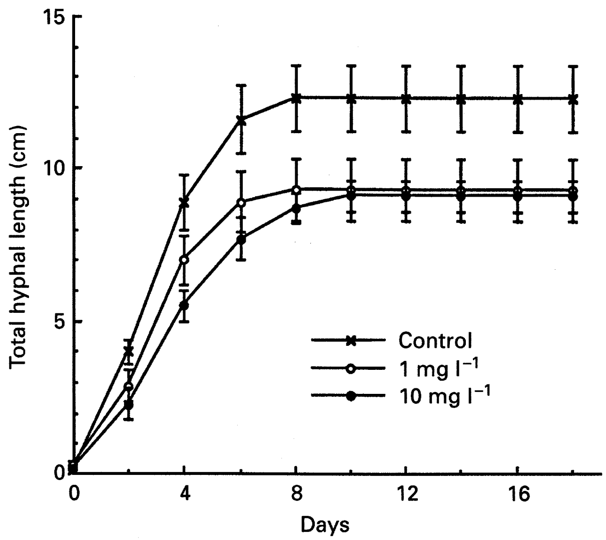

Figure 1. Effect of 0, 1, 2·5 and 10 mg l^{-1} *p*-hydroxybenzoic acid upon the hyphal growth from spores of *Gigaspora margarita* (means of 5 or 6 ± SEM).

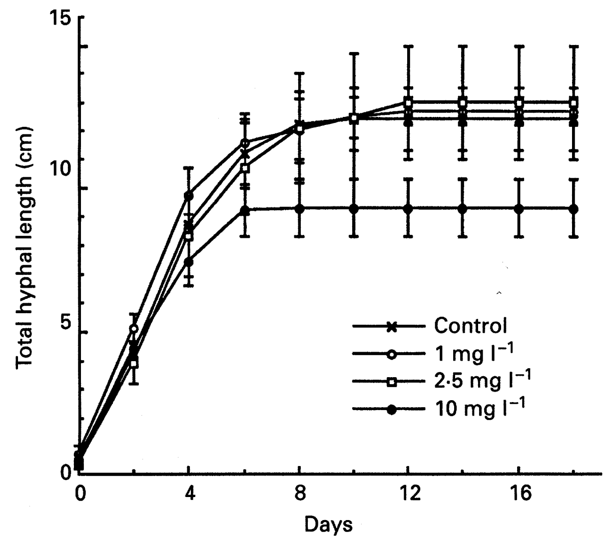

Figure 2. Effect of 0, 1, 2·5 and 10 mg l^{-1} ferulic acid upon the hyphal growth from spores of *Gigaspora margarita* (means of 5 ± SEM).

Effects of purified cell walls on hyphal growth

The cell walls from carrot and sugar beet roots had no significant effect on the growth of germinated *G. gigantea* spores (data not shown). Similarly, cell walls from carrot had no effect on the growth of *G. margarita* but sugar beet cell walls retarded the growth of this species (Table 3). The overall growth of *G. margarita* was inhibited by 37% and the number of branches off the primary germ tube were reduced by 62%.

Effects of phenolic compounds on hyphal growth

Carrot cell walls contain *p*-hydroxybenzoic acid which is not found in sugar beet cell walls (Nagahashi *et al.*, 1996). This compound had no effect on hyphal growth of *G. gigantea* at 5 mg l^{-1} (Table 4). A stimulation of growth of up to 60% was observed with increasing concentration of *p*-hydroxybenzoic acid when *G. margarita* was used in the bioassay (Table 5, Fig. 1). This phenolic also stimulated the production of a second germ tube at 2·5 mg l^{-1}. A second germ tube was produced in about 70% of the spores with 5 m l^{-1} and 10 mg l^{-1}. Only one spore in nine produced a second germ tube in the controls.

Ferulic acid, a major constituent of sugar beet cell walls and present also in carrot root cell walls (Nagahashi *et al.*, 1996), inhibited primary germ-tube growth of *G. gigantea* by 23% but had no significant effect on overall hyphal growth (Table 4). Both the growth of the primary germ tube of *G. margarita* and the overall hyphal length were inhibited by 16% and 25%, respectively, by

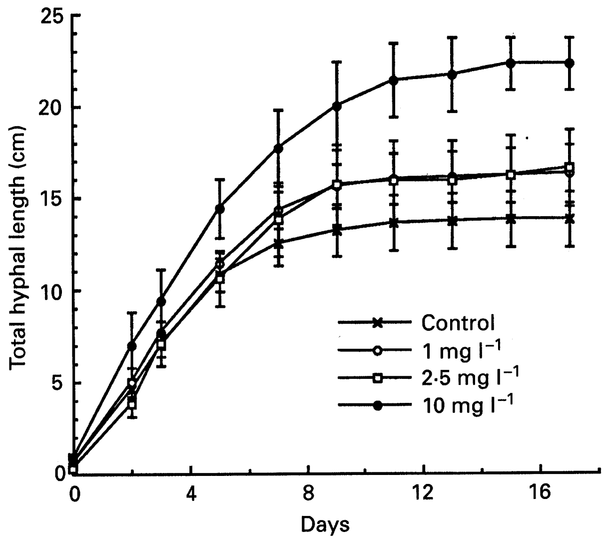

Figure 3. Effect of 0, 1, and 10 mg l^{-1} caffeic acid upon the hyphal growth from spores of *Gigaspora margarita* (means of 4 or 5 ± SEM).

10 mg l^{-1} ferulic acid (Table 5, Fig. 2). Ferulic acid caused the growth pattern of the primary germ tube to shift from straight to wavy at 5 mg l^{-1} in *G. gigantea* and at 10 mg l^{-1} in *G. margarita*.

Caffeic acid, found in the cytoplasm of carrot roots (Nagahashi *et al.*, 1996), significantly decreased total hyphal length, germ-tube length, and auxiliary-cell production in *G. gigantea* (Table 4). It also decreased the total hyphal growth of *G. margarita*, but had little effect on germ-tube growth or auxiliary cell production (Table 5, Fig. 3). Vanillic acid, found in carrot root cell-wall extracts (Nagahashi *et al.*, 1996), significantly increased overall hyphal growth (Table 4) and branching (6·2 ± 0·5 *vs.* 3·1 ± 0·7 for controls) of *G. gigantea* and primary germ-tube length (Table 5) and branching (8·8 ± 0·6 for controls *vs.* 15·6 ± 2·7 and 13·1 ± 2·4 for 0·1 and 10 mg l^{-1} vanillic acid, respectively) in *G. margarita*.

Since *G. gigantea* and *G. margarita* differed in their responses to the phenolics and extracts tested, the effect of quercetin, a flavonoid known to stimulate growth of AM fungi, was studied here. Quercetin stimulated the growth of *G. margarita* (Table 6), as reported earlier (Bécard *et al.*, 1992), but had no effects upon the growth of *G. gigantea*.

DISCUSSION

The observation that root penetration by AM fungi occurs at sites where secondary roots have initiated or started to emerge suggests a role for cell-wall components in the AM fungus–host interaction. The emerging secondary root secretes hydrolytic enzymes and esterases into the cell wall as it digests its way through the cortex and epidermis of the primary root. Covalently-bound compounds which stimulate hyphal growth could be released. We examined host (carrot) and non-host (sugar beet) Ri T-DNA-transformed roots to determine whether phenolics which are associated with plant cell walls and cytoplasm or cell walls themselves stimulate hyphal growth or signal the presence of a compatible root. We found that host-root factors were not always stimulatory and that non-host-root factors were not always inhibitory to the growth of two AM fungi. In addition, effects of these factors upon the two fungi studied differed in several instances.

Caffeic acid and sugar beet cytoplasmic extracts had inhibitory effects upon hyphal growth of both fungi. This is consistent with the observation that asparagus grown in sand treated with caffeic acid became less colonized by an AM fungus than did controls (Pedersen *et al.*, 1991). Ferulic acid and the carrot cell-wall extracts were the only solutions tested which induced gross changes in hyphal growth (change from straight to wavy growth). Ferulic acid also was shown to lessen colonization of asparagus by AM fungi (Wacker, Safir & Stephens, 1990; Pedersen *et al.*, 1991) and decrease hyphal elongation of *Glomus fasciculatum* (Wacker *et al.*, 1990).

Para-hydroxybenzoic acid, only associated with the host cell walls, stimulated hyphal growth at the highest concentrations used. Vanillic acid, also only associated with walls of host-root cells, stimulated hyphal growth and branching of both fungi tested. Increased hyphal growth and branching should enhance the probability of contact with a host root. Ferulic acid, a major phenolic of the non-host root-cell walls and present in host cell walls, had significant negative effects upon the overall length of hyphae produced by the two fungi when tested at high concentrations. These results are consistent with a role for specific cell wall-associated com-

Table 6. *Effect of 10 μM quercetin upon growth of hyphae from spores of* Gigaspora gigantea *and* Gigaspora margarita *after 18 d**

Treatment	*Gigaspora gigantea* Total (cm)	*Gigaspora gigantea* Germ tube (cm)	*Gigaspora margarita* Total (cm)	*Gigaspora margarita* Germ tube (cm)
Control	54·4 ± 4·3	14·8 ± 0·3	18·9 ± 2·7	4·6 ± 0·9
Quercetin	45·5 ± 4·7	13·9 ± 0·2	44·8 ± 7·1	13·5 ± 2·2

* Means of 4–6 ± SEM.

pounds during fungal growth prior to colonization. However, we do not know the physiological concentrations of these compounds, and some cell wall-associated phenolics have yet to be identified and assayed for their effects upon AM fungi (Nagahashi *et al.*, 1996). There is no evidence, however, that colonization of roots by AM fungi induces changes in cell wall-bound phenolics that would affect hyphal growth (Codignola *et al.*, 1989; Maier *et al.*, 1995).

These results also demonstrate the need for caution when interpreting bioassays of crude extracts. This was further confirmed by the inhibition of growth caused by caffeic acid which was found in the carrot cell cytoplasm. Compounds which stimulate and inhibit growth of AM fungi might both be present in crude extracts and counteract each other's effect in the bioassay. Cell wall-associated components might first have to be identified so that individual compounds can be tested in the bioassay, as was done in this study, or crude extracts may have to be fractionated before use.

Several mechanisms have been proposed to explain why some plants do not become colonized by AM fungi (Tester *et al.*, 1987). They might exude inhibitory substances such as isothiocyanates (Schreiner & Koide, 1993), lack stimulatory substances in their exudates (Bécard & Piché, 1990), or exhibit physical or chemical characteristics in epidermal cells which prohibit differentiation of, or growth from, the infection peg (Tester *et al.*, 1987). We found that phenolics associated with the cell walls of sugar beet roots did not affect fungal growth to the point that the hyphae would be prohibited from contacting a root.

Finally, the different responses of *G. gigantea* and *G. margarita* to various growth-medium amendments underscore the importance of testing a variety of AM fungi when studying hyphal growth responses to root signals.

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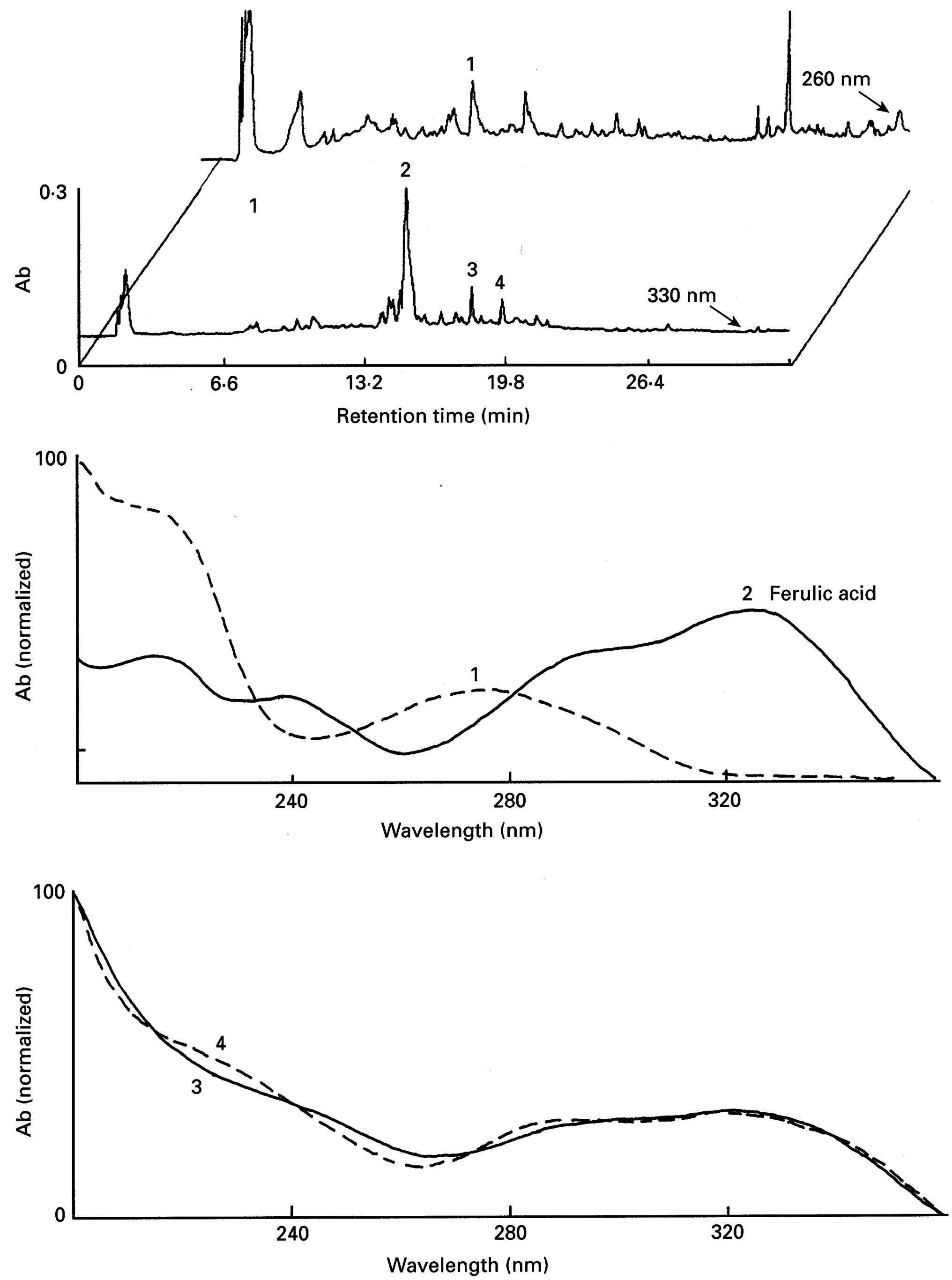

Figure 6. HPLC separation of compounds which were alkaline-hydrolysed from cytoplasmic conjugates of sugar beet roots before extraction in butanol. See text for details.

time of 18·8 min was also present in the cell-wall extract of sugar beet roots (cf. Figs 3, 6).

DISCUSSION

If the cell walls are a source of signals that are involved in recognition events, stimulation of hyphal growth, and possibly appressorium formation, then the cell walls of a host plant should show inherent differences from those of a non-host plant. This study showed that the carrot cell wall has at least four phenolic compounds (including *p*-HBA and vanillic acid) which are not present in sugar beet cell walls. Furthermore, the sugar beet cell wall has at least four compounds (retention times of 15·4, 17·1, 18·0 and 18·8 min) which are not present in carrot root cell walls.

To complete the comparative study, the cytoplasmic fractions from sugar beets and carrots were also compared. Caffeic acid, and an unidentified peak with retention time of 8 min were unique to the carrot cytoplasm. Five compounds with retention times of 11·3, 14·3, 17·1, 18·8, 19·5 min were only found in the sugar beet cytoplasm.

The results reported here are significant and relevant to future research on host–AM fungi interactions. Carrot roots contain unique phenolics both in the cell wall and in the cytoplasm. Similarly, sugar beet roots contain unique phenolic compounds extracellularly and intracellularly. In our subsequent paper (Douds *et al.*, 1996), the efficacy of the identified phenolics on the growth of germinated AM fungal spores is tested. This approach would be useful in determining what makes a root a host or non-host for mycorrhizal association.

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